NUCLEOTIDE SEQUENCES DERIVED FROM GENES CODING FOR TRIMETHYLAMINE N-OXIDE REDUCTASE, AND USES THEREOF, ESPECIALLY FOR THE DETECTION OF BACTERIA

The present invention relates to nucleotide sequences derived from genes coding for trimethylamine N-oxide reductase (TMAO reductase) and which can be used, especially as primers, for implementing methods of detecting bacteria involved in spoilage of the flesh of aquatic animals, and more particularly of marine animals.

Trimethylamine N-oxide (TMAO) is one of the main constituents of low molecular weight in marine animals, where it can represent up to 1% of their dry weight. In anaerobic conditions, certain bacteria utilize TMAO as an exogenous electron acceptor for their respiration. TMAO is then reduced to trimethylamine (TMA), a highly volatile compound that is largely responsible for the foul-smelling odour of decomposing fish. Reduction of TMAO to TMA has been demonstrated in marine bacteria, such as those of the genus *Shewanella*, *Photobacterium* or *Vibrio* (1, 2), in photosynthetic bacteria isolated in brackish water, such as those of the genus *Rhodobacter* (3), but also in several enterobacteria, such as *Escherichia coli, Salmonella typhimurium* or *Proteus vulgaris* (4).

The enzyme responsible for the reduction of TMAO to TMA corresponds to trimethylamine N-oxide reductase (TMAO reductase), the properties of which have been investigated in various organisms. As an example, in *E. coli* there are two separate enzymes responsible for the reduction of TMAO: TMAO reductase and DMSO reductase. TMAO reductase, which is responsible for 90% of the reduction of TMAO, is a periplasmic molybdoenzyme of 90 kDa, which can occur in monomeric or dimeric form (5). The TMAO reductases of the various bacteria investigated are all molybdoenzymes of 80-100 kDa that may occur as monomer, dimer or tetramer. These proteins can all be induced in anaerobiosis by TMAO, but also by DMSO (dimethyl sulphoxide). Finally, except in *P. vulgaris*, the TMAO reductases are all localized in the bacterium's periplasm.

The TMAO reductase system has been investigated at the genetic and molecular level in the enterobacteria *E. coli* and those of the genus *Rhodobacter*. The two TMAO/DMSO reductases of bacteria of the genus *Rhodobacter* and the inducible TMAO reductase of *E. coli* display homology at the level of their primary sequence (6,

7, 8). The overall structure of the TMAO reductase of *E. coli* might be similar to those described for *Rhodobacter sphaeroides* or *Rhodobacter capsulatus*. In *E. coli*, the genes that code for the various components of the TMAO reductase system are organized as an operon: the *torCAD* operon, which codes for the three proteins TorC, TorD and TorA (6). In *Rhodobacter*, the genes that code for the various components of the TMAO/DMSO reductase system are also organized as an operon: the *dorCDA* operon, which codes for the three proteins DorC, DorD (also called DorB) and DorA, possessing homologies with, respectively, the proteins TorC, TorD and TorA of *E. coli* (9, 10).

A phylogenetic study by sequencing of rDNA 16S was conducted on a bacterium isolated from a fish caught in the Bay of Marseilles, and stored at room temperature in filtered sea water. The results obtained show that this bacterium, which possesses considerable TMAO reductase activity, corresponds to a new species of the genus *Shewanella*, close to *Shewanella putrefaciens*. It has been named *Shewanella massilia*. Apart from the TorE protein, the products of the genes of the *torECAD* operon of *S. massilia* have strong homology with the proteins involved in the TMAO reductase systems of *E. coli* and of the bacteria of the genus *Rhodobacter* (19). Moreover, the overall structure of the TMAO reductase of *S. massilia* exhibits homologies with the structures already described of the TMAO/DMSO reductases of the bacteria of the genus *Rhodobacter* (20).

Spoilage of the flesh of fish, as with most foodstuffs, is essentially due to the metabolic activity of certain bacteria, which may represent up to 10^8 cells per gram of product. The first changes that occur *post mortem* in the flesh of fish are not necessarily due to bacteria, but to certain reactions of autolysis. However, microbial development very quickly constitutes the main element in spoilage, as the muscle tissue of the fish provides a substrate for growth of bacteria. Several factors that are intrinsic to the flesh of fish can explain their ease of spoilage, compared with other foodstuffs: considerable hydration, a high *post mortem* pH (> 6), a high content of non-protein nitrogen-containing substances, mostly TMAO, and a low content of structural proteins (11, 12). These various characteristics contribute to the development of a degradative microbial flora that is specific to the flesh of fish. Interestingly, the diversity of fish species does not seem to have an influence on the nature or the number of bacteria initially present on the fish. On the other hand, the bacterial microflora of the fish seems to be directly related to the natural environment where it evolves. Thus, contamination of the water,

which depends essentially on the temperature, salinity or the concentration of dissolved oxygen, seems to play a decisive role. The bacteria found in the flesh of fish do not originate solely from the microflora of living fish: contaminating bacteria may originate in particular from the refrigeration procedure (ice, fish containers, processing steps, etc.). Among all of the microbial flora found in the flesh of fish, only a proportion of them have been held to be responsible for the phenomenon of putrefaction. Although bacterial contamination does not necessarily make fish unfit for consumption, certain bacteria quickly lead to unpleasant changes in texture, taste or odour (13).

Thus, seafoods are highly perishable products, giving the fishing industry many storage problems. Controlling the freshness of these products is an increasingly important concern for the entire fishery sector (producers, processors or distributors), and for some years the industry has been trying to find new techniques for determining the freshness of fish in very short times.

At present, determination of the freshness of seafoods is based essentially on sensory (organoleptic) appraisal (appearance, odour, taste), which is best for judging whether a product is fresh, moderately fresh or unfit for consumption. This organoleptic method is, however, only applicable to whole products, and more reliable laboratory analyses are often indispensable.

Methods based on bacteriological analysis (total bacterial flora), applicable to processed products, are being used more and more for assessing the freshness of fish. Direct determination of the number of bacteria that can be grown on a sample of fish is still the most commonly used method: after growing on various media (for example iron citrate medium), each cell will form a colony that is visible in Petri dishes, and so can be counted. Although this technique is easy to use and is quite sensitive, it requires a considerable incubation time for growth (about 3 to 5 days). Furthermore, the composition of the growth medium, or the temperatures adopted for these tests (20-30°C), do not always make it possible to detect all of the degrading bacteria. Thus, existing regulations that specify a temperature of 30°C for conducting these tests (decree of 1979) do not permit the detection of certain degrading bacteria such as *Photobacterium phosphoreum*, which do not grow at that temperature. Thus, even if nonspecific measurement of the total microbial burden constitutes a good indicator of the hygienic state of fish, it cannot be used for evaluating the organoleptic quality of this type of product. Microscopic examination of foodstuffs is a rapid method of determining the level of bacterial contamination. Cells stained with acridine orange are

visualized by a technique of fluorescence microscopy; the main shortcoming of this method is, however, its low sensitivity (between 10^4 and 10^5 cells).

Degradation of the cellular constituents of fish leads to the production of a great many substances that can serve as an indirect indicator of bacterial contamination. An alternative to the methods mentioned above therefore consisted of using chemical techniques for monitoring various products of degradation of fish flesh (14). Determination of total volatile basic nitrogen (TVBN), consisting mostly of ammonia (NH_3) and trimethylamine (TMA), is a technique that is now widely employed for determining the degree of decomposition of fish flesh. Determination of TVBN has the advantage of being simple, quick and of very low cost. Determination of the proportion of TMA in TVBN can add an additional qualitative element to the TVBN criterion. This criterion in fact seems less dependent on variations that generally affect the proportion of TMA or TVBN (the species of fish, its fats content etc.). The same methodology as employed for TVBN can be used for determining TMA. Other colorimetric, enzymatic or chromatographic techniques have also been described for quantitative determination of TMA (15, 16). During the reaction of reduction of TMAO to TMA by bacteria, decomposing fish flesh undergoes various changes: lowering of the redox potential, increase in pH and electrical conductivity. These various indicators have also been employed in several studies with the aim of monitoring the release of TMA indirectly. However, none of these studies has ever established a definite correlation between the level of TMA released and the freshness of fish samples. A great many other degradation products have also been tested with a view to determining the extent of spoilage of fish using chemical determinations: hydrogen sulphide, hypoxanthine, indole, histamine and amino acids. However, these various determinations are only significant for certain species of fish, or when spoilage is very advanced.

The polymerase chain reaction (PCR) is a molecular technique for amplifying large quantities of a target DNA sequence for its visualization. For some years, the PCR technique has been used increasingly for detecting and identifying microorganisms, notably in the field of clinical bacteriology or food safety (17, 18). At present, PCR tests applied to foodstuffs mainly provide the detection of well-defined pathogenic bacteria (*Shigella*, *Salmonella*, *Listeria*, etc.).

The present invention follows from the demonstration, by the inventors, that the DNA sequences coding for a protein of the TMAO reductase system have a sufficient homology among various bacteria, especially among the bacteria responsible for the

degradation of tissues of aquatic organisms, and more particularly among marine bacteria, to permit the application of methods of detection of all bacteria responsible for tissue degradation by their TMAO reductase activity, the said methods being applicable to any aquatic animal, and especially to any marine animal.

One of the aims of the present invention is to provide a test of the freshness of aquatic products, and more particularly of seafoods, that is both rapid and inexpensive, and offers increased reliability in terms of specificity and sensitivity.

One of the other aims of the present invention is to provide a test for detecting not one type of bacteria, but all of the bacteria responsible for the degradation of tissues of aquatic organisms, and especially the putrefaction of fish flesh, namely both marine bacteria and enterobacteria or the bacteria isolated in brackish water.

Another aim of the present invention is to provide a test for detecting bacteria responsible for the degradation of the tissues of marine organisms, applicable to various species of fish from various geographical regions.

Another aim of the present invention is to provide a test for detecting bacteria responsible for the degradation of tissues of marine organisms, that is sufficiently sensitive for detecting early stages of degradation.

The present invention relates to the use of nucleotide sequences chosen from those comprising a sequence coding for a protein of the trimethylamine N-oxide reductase system (TMAO reductase) in bacteria, or a fragment of this sequence such as a probe or a primer of about 15 to about 25 nucleotides, or a sequence derived by addition, suppression, and/or substitution of one or more nucleotides of this sequence coding for a protein of the TMAO reductase system or a fragment of the latter, the said fragment and the said derived sequence being capable of hybridizing, especially in the conditions of hybridization defined below, with the said sequence coding for a protein of the TMAO reductase system, for the application of a method of detecting the presence of all bacteria involved in the process of degradation of the flesh of aquatic animals, in a host that can be a carrier of these bacteria.

Advantageously, the aforementioned nucleotide sequences coding for a protein of the TMAO reductase system are chosen from those corresponding to the *tor*CAD operon or the *dor*CDA operon mentioned above, of the TMAO reductase system in bacteria.

Preferably, the aforementioned sequence coding for a protein of the aforementioned TMAO reductase system is chosen from the sequences coding for

TMAO reductase in bacteria, further designated TorA or DorA protein, or coding for a type *c* cytochrome in bacteria, further designated TorC or DorC protein.

According to a particularly advantageous embodiment of the invention, the nucleotide sequences coding for a protein of the TMAO reductase system are chosen from those of the following bacteria:

– marine bacteria, such as those of the genus *Shewanella*, *Photobacterium* or *Vibrio*, the said sequences being chosen in particular from the following:

* the sequence SEQ ID NO:1 coding for the TorA protein of *Shewanella massilia* shown in Figure 1,

* the sequence SEQ ID NO:2 coding for the TorA protein of *Shewanella putrefaciens* shown in Figure 1,

* the sequence SEQ ID NO:3 coding for the TorA protein of *Shewanella c* shown in Figure 2,

* the partial sequence SEQ ID NO:4 coding for the TorA protein of *Photobacterium phosphoreum* shown in Figure 3,

* the sequence coding for the TorC protein SEQ ID NO:5 of *Shewanella massilia* shown in Figure 14,

– bacteria obtained from brackish water, such as those of the genus *Rhodobacter*, or *Roseobacter*, the said sequences being chosen in particular from the following:

* the sequence SEQ ID NO:6 coding for the DorA protein of *Rhodobacter sphaeroides* shown in Figure 4,

* the sequence SEQ ID NO:7 coding for the DorA protein of *Rhodobacter capsulatus* shown in Figure 4,

* the sequence coding for the DorC protein SEQ ID NO:8 of *Rhodobacter sphaeroides* shown in Figure 14,

– enterobacteria, such as those of the genus *Escherichia*, or *Salmonella*, the said sequences being chosen in particular from the following:

* the sequence SEQ ID NO:9 coding for the TorA protein of *Escherichia coli* shown in Figure 4,

* the partial sequence SEQ ID NO:10 coding for the TorA protein of *Salmonella typhimurium* shown in Figure 5,

* the sequence coding for the TorC protein SEQ ID NO:11 of *Escherichia coli* shown in Figure 14.

The invention relates more particularly to the aforementioned use of nucleotide sequences chosen from the fragments of the sequences defined above, or from sequences derived from these fragments, the said fragments or derived sequences comprising about 15 to 25 nucleotides, and being used in pairs for forming pairs of primers permitting the amplification of fragments of genes coding for a protein of the TMAO-reductase system of bacteria involved in the degradation of the flesh of aquatic animals, according to the PCR technique.

Advantageously, the aforementioned nucleotide sequences are used in the form of pairs of primers chosen from any one of the following three groups of primers:

(1) the group of primers "DDN" amplifying DNA fragments of the *tor*A gene, the said group comprising:

- the following compositions of nucleotide sequences "DDN+":

  – DDN1+ (SEQ ID NO:12): *5'* CGG vGA yTA CTC bAC hGG TGC *3'* : mixture of 54 nucleotide sequences,

  – DDN5+ (SEQ ID NO:13): *5'* ATy GAT GCG ATy CTC GAA CC *3'* : mixture of 4 nucleotide sequences,

- the following compositions of nucleotide sequences "DDN-":

  – DDN2- (SEQ ID NO:14): *5'* CGT Amw sGT CGA kAT CGT TrC GCT C *3'* : mixture of 32 nucleotide sequences,

  – DDN3- (SEQ ID NO:15): *5'* GAC TCA CAy Awy TGy GAG TG *3'* : mixture of 16 nucleotide sequences,

  – DDN4- (SEQ ID NO:16): *5'* TGr CCd CGr kCG TTA AAG AC *3'* : mixture of 24 nucleotide sequences,

  – DDN5- (SEQ ID NO:17): *5'* CCv GGT TCG AGr ATC GCA TC *3'* : mixture of 6 nucleotide sequences,

(2) the group of primers "BN" amplifying DNA fragments of the *tor*A gene, the said group comprising:

- the following compositions of nucleotide sequences "BN+":

  – BN1+ (SEQ ID NO:18): *5'* C bGA yAT CsT rCT GCC *3'* : mixture of 16 nucleotide sequences,

  – BN3+ (SEQ ID NO:19): *5'* GGm GAy TAy TCb ACm GGy GC *3'* : mixture of 96 nucleotide sequences,

– BN6+ (SEQ ID NO:20): *5'* Twy GAr CGy AAC GAy mTC GA *3'* : mixture of 64 nucleotide sequences,

◆ the following compositions of nucleotide sequences "BN-":

– BN2- (SEQ ID NO:21): *5'* GG vyC rTA CCA bsC vCC TTC *3'* : mixture of 216 nucleotide sequences,

– BN4- (SEQ ID NO:22): *5'* ATC Arr CCn swv GGC GTG CC *3'* : mixture of 192 nucleotide sequences,

– BN5- (SEQ ID NO:23): *5'* GbC ACr TCd GTy TGy GG *3'* : mixture of 72 nucleotide sequences,

(3) the group of primers "BC" amplifying DNA fragments of the *tor*C gene, the said group comprising:

◆ the following compositions of nucleotide sequences "BC+":

– BC1+ (SEQ ID NO:24): *5'* ACn CCn GAr AAr TTy GAr GC *3'* : mixture of 256 nucleotide sequences,

– BC2+ (SEQ ID NO:25): *5'* TGy ATh GAy TGy CAy AAr GG *3'* : mixture of 96 nucleotide sequences,

◆ the following compositions of nucleotide sequences "BC-":

– BC2- (SEQ ID NO:26): *5'* CCy TTr TGr CAr TCd ATr CA *3'* : mixture of 96 nucleotide sequences,

– BC3- (SEQ ID NO:27): *5'* TTn GCr TCr AAr TGn GC *3'* : mixture of 128 nucleotide sequences,

in which n = (A,C,G,T), y = (C,T), r = (A,G), h = (A,C,T), d = (G,A,T), m = (A,C), w = (A,T), b = (G,T,C), s = (G,C), v = (G,A,C), and k = (G,T),

the pairs of primers being chosen in such a way that one of the primers of a pair corresponds to one of the aforementioned compositions of nucleotide sequences DDN+, BN+ or BC+, whereas the other primer corresponds respectively to one of the aforementioned compositions of nucleotide sequences DDN-, BN- or BC-, the said pairs of primers being chosen in particular from any one of the following four pairs:

(a) the pair DDN1+/DDN5-, leading to amplification of fragments of the gene coding for the TorA protein in bacteria, of a size of about 820 base pairs (bp), and especially to the amplification of an 821 bp fragment of the gene coding for the TorA protein in bacteria of the genus *Shewanella*, such as the 821 bp fragment bounded by

the nucleotides located in positions 620 to 1450 of the *tor*A gene of *S. massilia* shown in Figure 4,

(b) the pair BN6+/BN2-, leading to amplification of fragments of the gene coding for the TorA protein in bacteria, with a size of about 710 bp, and especially to the amplification of a 727 bp fragment of the gene coding for the TorA protein in bacteria of the genus *Shewanella*, such as the 727 bp fragment bounded by the nucleotides located in positions 1657 to 2403 of the *tor*A gene of *S. massilia* shown in Figure 4,

(c) the pair BN6+/BN4-, leading to amplification of fragments of the gene coding for the TorA protein in bacteria, with a size of about 360 bp, and especially to the amplification of a 355 bp fragment of the gene coding for the TorA protein in bacteria of the genus *Shewanella*, such as the 355 bp fragment bounded by the nucleotides located in positions 1657 to 2023 of the *tor*A gene of *S. massilia* shown in Figure 4,

(d) the pair BC1+/BC2-, leading to amplification of fragments of the gene coding for the TorC protein in bacteria, with a size of about 170 bp, and especially to the amplification of a 197 bp fragment of the gene coding for the TorC protein in bacteria of the genus *Shewanella*, such as the 197 bp fragment coding for the polypeptide fragment bounded by the amino acids located in positions 114 to 179 of the TorC protein of *S. massilia* shown in Figure 14.

The hosts that can be carriers of bacteria involved in the process of degradation of the flesh of aquatic animals, such as those described above, are aquatic organisms, in particular marine organisms such as fish and crustacea, and more particularly Atlantic fish such as sole and cod, or fish from the Mediterranean Sea such as surmullet and sea bream, as well as certain animals from fresh water or brackish water.

The invention relates more particularly to the aforementioned use of nucleotide sequences described above, for implementing a method of detecting the presence of all bacteria involved in the degradation of the flesh of aquatic animals, within the framework of a method of evaluating the state of freshness of aquatic animals from which the test sample was obtained, when the latter are removed from their natural environment.

The invention also relates to any nucleotide sequence corresponding to one of the following sequences:

- DDN1+ : *5'* CGG vGA yTA CTC bAC hGG TGC *3'*,
- DDN5+ : *5'* ATy GAT GCG ATy CTC GAA CC *3'*,
- DDN2- : *5'* CGT Amw sGT CGA kAT CGT TrC GCT C *3'*,

– DDN3- : *5'* GAC TCA CAy Awy TGy GAG TG *3'*,

– DDN4- : *5'* TGr CCd CGr kCG TTA AAG AC *3'*,

– DDN5- : *5'* CCv GGT TCG AGr ATC GCA TC *3'*,

– BN1+ : *5'* C bGA yAT CsT rCT GCC *3'*,

– BN3+ : *5'* GGm GAy TAy TCb ACm GGy GC *3'*,

– BN6+ : *5'* Twy GAr CGy AAC GAy mTC GA *3'*,

– BN2- : *5'* GG vyC rTA CCA bsC vCC TTC *3'*,

– BN4- : *5'* ATC Arr CCn swv GGC GTG CC *3'*,

– BN5- : *5'* GbC ACr TCd GTy TGy GG *3'*,

– BC1+ : *5'* ACn CCn GAr AAr TTy GAr GC *3'*,

– BC2+ : *5'* TGy ATh GAy TGy CAy AAr GG *3'*,

– BC2- : *5'* CCy TTr TGr CAr TCd ATr CA *3'*,

– BC3- : *5'* TTn GCr TCr AAr TGn GC *3'*,

in which n = (A,C,G,T), y = (C,T), r = (A,G), h = (A,C,T), d = (G,A,T), m = (A,C), w = (A,T), b = (G,T,C), s = (G,C), v = (G,A,C), and k = (G,T).

The invention also relates to any composition of a mixture of nucleotide sequences, corresponding to one of the following compositions:

◆ the following compositions of nucleotide sequences "DDN+":

– DDN1+ : *5'* CGG vGA yTA CTC bAC hGG TGC *3'* : mixture of 54 nucleotide sequences,

– DDN5+ : *5'* ATy GAT GCG ATy CTC GAA CC *3'* : mixture of 4 nucleotide sequences,

◆ the following compositions of nucleotide sequences "DDN-":

– DDN2- : *5'* CGT Amw sGT CGA kAT CGT TrC GCT C *3'* : mixture of 32 nucleotide sequences,

– DDN3- : *5'* GAC TCA CAy Awy TGy GAG TG *3'* : mixture of 16 nucleotide sequences,

– DDN4- : *5'* TGr CCd CGr kCG TTA AAG AC *3'* : mixture of 24 nucleotide sequences,

– DDN5- : *5'* CCv GGT TCG AGr ATC GCA TC *3'* : mixture of 6 nucleotide sequences,

◆ the following compositions of nucleotide sequences "BN+":

– BN1+ : *5'* C bGA yAT CsT rCT GCC *3'* : mixture of 16 nucleotide sequences,

– BN3+ : *5'* GGm GAy TAy TCb ACm GGy GC *3'* : mixture of 96 nucleotide sequences,

– BN6+ : *5'* Twy GAr CGy AAC GAy mTC GA *3'* : mixture of 64 nucleotide sequences,

◆ the following compositions of nucleotide sequences "BN-":

– BN2- : *5'* GG vyC rTA CCA bsC vCC TTC *3'* : mixture of 216 nucleotide sequences,

– BN4- : *5'* ATC Arr CCn swv GGC GTG CC *3'* : mixture of 192 nucleotide sequences,

– BN5- : *5'* GbC ACr TCd GTy TGy GG *3'* : mixture of 72 nucleotide sequences,

◆ the following compositions of nucleotide sequences "BC+":

– BC1+ : *5'* ACn CCn GAr AAr TTy GAr GC *3'* : mixture of 256 nucleotide sequences,

– BC2+ : *5'* TGy ATh GAy TGy CAy AAr GG *3'* : mixture of 96 nucleotide sequences,

◆ the following compositions of nucleotide sequences "BC-":

– BC2- : *5'* CCy TTr TGr CAr TCd ATr CA *3'* : mixture of 96 nucleotide sequences,

– BC3- : *5'* TTn GCr TCr AAr TGn GC *3'* : mixture of 128 nucleotide sequences,

in which n = (A,C,G,T), y = (C,T), r = (A,G), h = (A,C,T), d = (G,A,T), m = (A,C), w = (A,T), b = (G,T,C), s = (G,C), v = (G,A,C), and k = (G,T).

The invention also relates to pairs of primers chosen from one of the following groups of primers:

(1) the group of primers "DDN" comprising:

◆ the following compositions of nucleotide sequences "DDN+":

– DDN1+ : *5'* CGG vGA yTA CTC bAC hGG TGC *3'* : mixture of 54 nucleotide sequences,

– DDN5+ : *5′* ATy GAT GCG ATy CTC GAA CC *3′* : mixture of 4 nucleotide sequences,

◆ the following compositions of nucleotide sequences "DDN-":

– DDN2- : *5′* CGT Amw sGT CGA kAT CGT TrC GCT C *3′* : mixture of 32 nucleotide sequences,

– DDN3- : *5′* GAC TCA CAy Awy TGy GAG TG *3′* : mixture of 16 nucleotide sequences,

– DDN4- : *5′* TGr CCd CGr kCG TTA AAG AC *3′* : mixture of 24 nucleotide sequences,

– DDN5- : *5′* CCv GGT TCG AGr ATC GCA TC *3′* : mixture of 6 nucleotide sequences,

(2) the group of primers "BN" comprising:

◆ the following compositions of nucleotide sequences "BN+":

– BN1+ : *5′* C bGA yAT CsT rCT GCC *3′* : mixture of 16 nucleotide sequences,

– BN3+ : *5′* GGm GAy TAy TCb ACm GGy GC *3′* : mixture of 96 nucleotide sequences,

– BN6+ : *5′* Twy GAr CGy AAC GAy mTC GA *3′* : mixture of 64 nucleotide sequences,

◆ the following compositions of nucleotide sequences "BN-":

– BN2- : *5′* GG vyC rTA CCA bsC vCC TTC *3′* : mixture of 216 nucleotide sequences,

– BN4- : *5′* ATC Arr CCn swv GGC GTG CC *3′* : mixture of 192 nucleotide sequences,

– BN5- : *5′* GbC ACr TCd GTy TGy GG *3′* : mixture of 72 nucleotide sequences,

(3) the group of primers "BC" comprising:

◆ the following compositions of nucleotide sequences "BC+":

– BC1+ : *5′* ACn CCn GAr AAr TTy GAr GC *3′* : mixture of 256 nucleotide sequences,

– BC2+ : *5′* TGy ATh GAy TGy CAy AAr GG *3′* : mixture of 96 nucleotide sequences,

◆ the following compositions of nucleotide sequences "BC-":

– BC2- : *5'* CCy TTr TGr CAr TCd ATr CA *3'* : mixture of 96 nucleotide sequences,

– BC3- : *5'* TTn GCr TCr AAr TGn GC *3'* : mixture of 128 nucleotide sequences,

in which n = (A,C,G,T), y = (C,T), r = (A,G), h = (A,C,T), d = (G,A,T), m = (A,C), w = (A,T), b = (G,T,C), s = (G,C), v = (G,A,C), and k = (G,T),

the pairs of primers being chosen in such a way that one of the primers of a pair corresponds to one of the aforementioned compositions of nucleotide sequences DDN+, BN+ or BC+, whereas the other primer corresponds respectively to one of the aforementioned compositions of nucleotide sequences DDN-, BN- or BC-, the said pairs of primers being chosen in particular from any one of the following four pairs:

(a) the pair DDN1+/DDN5-, leading to amplification of fragments of the gene coding for the TorA protein in bacteria, of a size of about 820 base pairs (bp), and especially to the amplification of an 821 bp fragment of the gene coding for the TorA protein in bacteria of the genus *Shewanella*, such as the 821 bp fragment bounded by the nucleotides located in positions 620 to 1450 of the *tor*A gene of *S. massilia* shown in Figure 4,

(b) the pair BN6+/BN2-, leading to amplification of fragments of the gene coding for the TorA protein in bacteria, with a size of about 710 bp, and especially to the amplification of a 727 bp fragment of the gene coding for the TorA protein in bacteria of the genus *Shewanella*, such as the 727 bp fragment bounded by the nucleotides located in positions 1657 to 2403 of the *tor*A gene of *S. massilia* shown in Figure 4,

(c) the pair BN6+/BN4-, leading to amplification of fragments of the gene coding for the TorA protein in bacteria, with a size of about 360 bp, and especially to the amplification of a 355 bp fragment of the gene coding for the TorA protein in bacteria of the genus *Shewanella*, such as the 355 bp fragment bounded by the nucleotides located in positions 1657 to 2023 of the *tor*A gene of *S. massilia* shown in Figure 4,

(d) the pair BC1+/BC2-, leading to amplification of fragments of the gene coding for the TorC protein in bacteria, with a size of about 170 bp, and especially to the amplification of a 197 bp fragment of the gene coding for the TorC protein in bacteria of the genus *Shewanella*, such as the 197 bp fragment coding for the polypeptide fragment bounded by the amino acids located in positions 114 to 179 of the TorC protein of *S. massilia* shown in Figure 14.

The invention also relates to a method of detecting all bacteria involved in the degradation of the flesh of aquatic animals in a host that can be a carrier of the said bacteria, the said method being effected starting from a biological sample taken from this host, this biological sample corresponding in particular to a subcutaneous fragment of flesh of the aquatic animal in question, and being characterized in that it comprises a step of hybridization of at least one nucleotide sequence as defined above, with fragments of genes coding for a protein of the TMAO-reductase system of bacteria involved in the degradation of the flesh of aquatic animals that can be present in the biological sample taken from the said host, followed by a step of detection, especially by electrophoresis, of the possible presence in the said sample of genes coding for a protein of the TMAO-reductase system, or of fragments of these genes, the number of copies of which has been amplified if necessary.

The invention relates more particularly to a method of detection as defined above, characterized in that it comprises the following steps:

– treatment of a biological sample taken from this host in order to extract the total DNA from this host and render the genome of these bacteria accessible to the nucleotide sequences or primers defined above, this treatment being effected in particular using a technique of rapid DNA extraction based on the fixation of nucleic acids to silica beads,

– amplification of the number of copies of genes coding for the proteins of the TMAO-reductase system of bacteria involved in the degradation of the flesh of aquatic animals, or of fragments of these genes, which can be present in this sample, by means of the aforementioned nucleotide sequences or primers,

– detection of the possible presence of an amplified number of copies of genes coding for a protein of the TMAO-reductase system of the aforementioned bacteria, or of fragments of these genes, and therefore of the presence of the said bacteria in the biological sample investigated.

Advantageously, the methods of detection according to the invention are characterized in that amplification of the number of genes coding for the proteins of the TMAO-reductase system comprises the following steps:

– predenaturation of the total double-stranded DNA of the host to single-stranded DNA, preferably in a buffer consisting of 10mM Tris-HCl pH 8.3, 50mM KCl, 1.5mM $MgCl_2$, 0.01% of gelatin, of the 4 deoxynucleotides constituting the DNAs (dCTP, dATP, dGTP, dTTP) each at a concentration of 100 μM, and of the pairs of primers as

defined above, by heating between about 90°C and about 100°C, advantageously at 94°C, for about 1.5 minute,

– actual amplification, by addition of DNA polymerase, for example Taq polymerase, to the medium obtained in the preceding step,

◆ heating to about 94°C for about 30 seconds, which corresponds to the actual denaturation step,

◆ then heating between about 35°C and about 60°C, and in particular at about 45°C or 55°C, for about 30 seconds, which corresponds to the step of hybridization of the primers with the genes coding for the proteins of the TMAO-reductase system of bacteria, or of the fragments of these genes, that can be present in the biological sample investigated,

◆ and finally, heating at 72°C, for about 45 seconds, which corresponds to the step of extension of the primers, hybridized in the preceding step, towards one other, thus producing complementary nucleotide sequences of fragments of genes coding for the proteins of the TMAO-reductase system of bacteria, these latter sequences being bounded by the nucleotides hybridizing with the aforementioned primers,

– repetition of the preceding amplification step between about 15 and about 35 times, advantageously about 30 times.

The invention also relates to kits for implementing a method of detection as mentioned above, characterized in that they comprise:

– one or more nucleotide sequences or primers as defined above,

– a DNA polymerase,

– a reaction medium preferably consisting of 10mM Tris-HCl pH 8.3, 50mM KCl, 1.5mM $MgCl_2$, 0.01% of gelatin, the 4 deoxynucleotides constituting the DNAs (dCTP, dATP, dGTP, dTTP) each at a concentration of 100 μM.

The invention also relates to any nucleotide sequence comprising:

– the sequence shown in Figure 2, of the torA gene coding for the TorA protein of the marine bacterium *Shewanella C*,

– or any sequence derived from the aforementioned sequence by degeneration of the genetic code, and coding for the TorA protein of *Shewanella C*, the peptide sequence of which is shown in Figure 6,

– or any sequence derived from the aforementioned nucleotide sequence, especially by substitution, suppression or addition of one or more nucleotides, the said

derived sequence preferably having a homology of about 35% to 100% with the aforementioned nucleotide sequence shown in Figure 2,

– or any fragment of the aforementioned nucleotide sequence, or of a sequence derived from the latter as defined above, the said fragment preferably being constituted of at least about 15 nucleotides.

The invention also relates to any peptide sequence coded by the aforementioned nucleotide sequence shown in Figure 2, and comprising:

– the amino acid sequence shown in Figure 6 of the TorA protein of *Shewanella c*,

– or a sequence derived from the aforementioned peptide sequence, especially by substitution, suppression or addition of one or more amino acids, the said derived sequence preferably having a homology of about 35% to 100% with the aforementioned peptide sequence shown in Figure 6,

– or a fragment of the aforementioned peptide sequence, or of a sequence derived from the latter as defined above, the said fragment preferably being constituted of at least about 5 amino acids.

The invention also relates to any nucleotide sequence comprising:

– the partial sequence shown in Figure 3, of the gene coding for the TorA protein of the marine bacterium *Photobacterium phosphoreum*,

– or any sequence derived from the aforementioned sequence by degeneration of the genetic code, and coding for the fragment of the TorA protein of *Photobacterium phosphoreum* whose peptide sequence is shown in Figure 7,

– or any sequence derived from the aforementioned nucleotide sequence, especially by substitution, suppression or addition of one or more nucleotides, the said derived sequence preferably having a homology of about 35% to 100% with the aforementioned nucleotide sequence shown in Figure 3,

– or any fragment of the aforementioned nucleotide sequence, or of a sequence derived from the latter as defined above, the said fragment preferably being constituted of at least about 15 nucleotides.

The invention also relates to any peptide sequence coded by the aforementioned nucleotide sequence shown in Figure 3, and comprising:

– the partial amino acid sequence shown in Figure 7, of the TorA protein of *Photobacterium phosphoreum*,

– or a sequence derived from the aforementioned peptide sequence, especially by substitution, suppression or addition of one or more amino acids, the said derived sequence preferably having a homology of about 35% to 100% with the aforementioned peptide sequence shown in Figure 7,

– or a fragment of the aforementioned peptide sequence, or of a sequence derived from the latter as defined above, the said fragment preferably being constituted of at least about 5 amino acids.

The invention also relates to any nucleotide sequence comprising:

– the partial sequence shown in Figure 5, of the gene coding for the TorA protein of the marine bacterium *Salmonella typhimurium*,

– or any sequence derived from the aforementioned sequence by degeneration of the genetic code, and coding for the TorA protein of *Salmonella typhimurium* whose peptide sequence is shown in Figure 8,

– or any sequence derived from the aforementioned nucleotide sequence, especially by substitution, suppression or addition of one or more nucleotides, the said derived sequence preferably having a homology of about 35% to 100% with the aforementioned nucleotide sequence shown in Figure 5,

– or any fragment of the aforementioned nucleotide sequence, or of a sequence derived from the latter as defined above, the said fragment preferably being constituted of at least about 15 nucleotides.

The invention also relates to any peptide sequence encoded by the aforementioned nucleotide sequence shown in Figure 5, and comprising:

– the amino acid sequence shown in Figure 8, of the TorA protein of *Salmonella typhimurium*,

– or a sequence derived from the aforementioned peptide sequence, especially by substitution, suppression or addition of one or more amino acids, the said derived sequence preferably having a homology of about 35% to 100% with the aforementioned peptide sequence shown in Figure 8,

– or a fragment of the aforementioned peptide sequence, or of a sequence derived from the latter as defined above, the said fragment preferably being constituted of at least about 5 amino acids.

The invention will be illustrated further with the aid of the following detailed description of the production of primers of the invention, and of their use for implementing a method of detection according to the invention.

Description of the drawings

– Figure 1 shows the alignment of the nucleotide sequences of the *tor*A genes of *Shewanella massilia* (torA/S.m.), of *Shewanella c* (torA/S.c.), and of *Shewanella putrefaciens* (torA/S.p.).

– Figure 2 shows the complete nucleotide sequence of the torA gene coding for the TMAO reductase of *Shewanella c.*

– Figure 3 shows the partial nucleotide sequence of the torA gene coding for the TMAO reductase of *Photobacterium phosphoreum.*

– Figure 4 shows the alignment of the nucleotide sequences of the *tor*A genes of *Shewanella massilia* (torA/S.m.), of *E. coli* (torA/E.c.), of *Rhodobacter sphaeroides* (TorA/R.s.), and *Rhodobacter capsulatus* (TorA/R.c.). The arrows indicate the position of the various primers on the *tor*A gene of the bacterium *Shewanella massilia*, produced starting from the conserved regions. More particularly, the positions of the various primers "DDN" and "BN" on the *tor*A gene of *S. massilia* are as follows:

DDN1+ : 620	BN1+ : 1628
DDN5+ : 1428	BN3+ : 621
DDN2- : 1684	BN6+ : 1657
DDN3- : 2201	BN2- : 2403
DDN4- : 2325	BN4- : 2023
DDN5- : 1450	BN5- : 946

– Figure 5 shows the partial nucleotide sequence of the torA gene coding for the TMAO reductase of *Salmonella typhimurium.*

– Figure 6 shows the complete peptide sequence of the TMAO reductase of *Shewanella c.*

– Figure 7 shows the alignment of the peptide sequences of the fragment of the TorA protein of *Photobacterium phosphoreum* (TorA/P.p.), deduced from the DNA fragment amplified in *P. phosphoreum* with the aid of DDN1+/DDN5- molecular primers, with the corresponding protein regions of the TorA proteins of *Shewanella massilia* (TorA/S.m.), of *E. coli* (TorA/E.c.), and of *Rhodobacter sphaeroides* (DorA/R.s.).

– Figure 8 shows the alignment of the peptide sequences of the fragment of the TorA protein of *Salmonella typhimurium* (TorA/S.t.), deduced from the DNA fragment amplified in *S. typhimurium* with the aid of DDN1+/DDN5- molecular primers, with the corresponding protein regions of the TorA protein of *E. coli* (TorA/E.c.), of the DorA

– Figure 13 shows the alignment of nucleic sequences of a highly conserved region of the gene that codes for the TMAO reductase of bacteria of the genus *Shewanella*, *Rhodobacter* and of *E. coli*, used for producing one of the "BN" molecular primers according to the invention.

(a) Approximate position and orientation of the various BN primers on the *torA* gene coding for the TMAO reductase of different bacteria of the genus *Shewanella*, *Rhodobacter* and *E. coli*,

(b) Example of strategy for production of one of the BN primers (BN3+); the framed zone corresponds to a highly conserved nucleic region of the gene that codes for the TMAO reductase of the bacteria *Shewanella massilia* (*torA/S. massilia*), of *E. coli*, of *Rhodobacter sphaeroides* (*dorA/R. sphaeroides*), and of *Rhodobacter capsulatus* (*dorA/R. capsulatus*).

– Figure 14 shows the alignment of protein sequences of the TorC cytochrome of *S. massilia* (TorC/S.m.), of *E. coli* (TorC/E.c.) and of the DorC cytochrome of *R. sphaeroides* (DorC/R.s.). The arrows indicate the position of the different primers on the *torC* gene of the bacterium *Shewanella massilia*, produced starting from conserved regions. As an example, the pair BC1+/BC2- leads to amplification of the 197 bp fragment of the gene coding for the TorC protein in *S. massilia*, the said fragment coding for the polypeptide of 66 amino acids bounded by the amino acids located in positions 114 and 179 of the peptide sequence of TorC of *S. massilia*. The pair BC1+/BC3- leads to amplification of the 719 bp fragment of the gene coding for the TorC protein in *S. massilia*, the said fragment coding for the polypeptide of 240 amino acids bounded by the amino acids located in positions 114 and 353 of the peptide sequence of TorC of *S. massilia*. The pair BC2+/BC3- leads to amplification of the 542 bp fragment of the gene coding for the TorC protein in *S. massilia*, the said fragment coding for the polypeptide of 181 amino acids bounded by the amino acids located in positions 173 and 353 of the peptide sequence of TorC of *S. massilia*.

– Figure 15 shows electrophoresis on agarose gel (2%) of the products from PCR when amplification is effected starting from the chromosomal DNA of *E. coli* (lane 1), of *S. typhimurium* (lane 2), of *P. phosphoreum* (lane 3), of *S. massilia* (lane 4), of *R. sphaeroides* (lane 5), or of *Erwinia chrysanthemi* (lane 6) using the molecular primers BC1+ and BC2-. The expected size of the DNA fragment (177 base pairs) is indicated by an arrow. Lanes M: size markers.

protein of *Rhodobacter sphaeroides* (DorA/R.s.), and of the TorA protein of *Shewanella massilia* (TorA/S.m.).

– Figure 9 shows the alignment of nucleic sequences of a highly conserved region of the gene that codes for the TMAO reductase of bacteria of the genus *Shewanella* and of *E. coli*, used for producing one of the "DDN" molecular primers according to the invention.

(a) Approximate position and orientation of the various DDN primers on the *torA* gene coding for the TMAO reductase of various bacteria of the genus *Shewanella* and of *E. coli*,

(b) Example of strategy for producing one of the DDN primers (DDN1+); the framed zone corresponds to a highly conserved nucleic region of the gene that codes for the TMAO reductase of bacteria of the genus *Shewanella* [*Shewanella massilia* (*torA/S. massilia*), *Shewanella putrefaciens* (*torA/S. putrefaciens*), *Shewanella c* (*torA/S.c.*)] and of *E. coli*.

– Figure 10 shows agarose gel electrophoresis of the products from PCR when amplification is effected starting from the chromosomal DNA of *Shewanella massilia* (lanes 1, 2, 3 and 4), of *Shewanella c* (lanes 5, 6, 7 and 8) and of *Shewanella putrefaciens* MR-1 (lanes 9, 10, 11 and 12) using the DDN1+/DDN5- molecular primers (lanes 1, 5 and 9; expected size: 820 bp), DDN5+/DDN2- (lanes 2, 6 and 10; expected size: 234 bp), DDN5+/DDN3- (lanes 3, 7 and 11; expected size: 740 bp), DDN5+/DDN4- (lanes 4, 8 and 12; expected size: 866 bp). Lanes M: size markers.

– Figure 11 shows agarose gel electrophoresis of the products from PCR when amplification is effected starting from the chromosomal DNA of *Photobacterium phosphoreum* using the pairs of primers DDN1+/DDN5- (lane 1; expected size: 820 bp), DDN5+/DDN2- (lane 2; expected size: 234 bp), DDN5+/DDN3- (lane 3; expected size: 740 bp) and DDN5+/DDN4- (lane 4; expected size: 866 bp). Lanes M: size markers.

– Figure 12 shows agarose gel electrophoresis of the products from PCR when amplification is effected starting from the chromosomal DNA of *S. massilia* (lanes 1, 4, 7 and 10), of *E. coli* (lanes 2, 5, 8 and 11) and of *S. typhimurium* (lanes 3, 6 and 9), using the pairs of molecular primers DDN1+/DDN5- (lanes 1 to 3; expected size: 820 bp), DDN5+/DDN2- (lanes 4 to 6; expected size: 234 bp), DDN5+/DDN3- (lanes 7 to 9; expected size: 740 bp), DDN5+/DDN4- (lanes 10 and 11; expected size: 866 bp). Lanes M: size markers.

– Figure 16 shows the influence of fish chromosomal DNA on the specificity of the molecular primers according to the invention. PCR amplification is effected using the molecular primers DDN1+ and DDN5- in the presence of 2.5 µg (lanes 1 and 4), 5 µg (lanes 2 and 5), or 10 µg (lanes 3 and 6) of purified herring chromosomal DNA. Lanes 4, 5 and 6 correspond to tests carried out with addition of 2.5 µl of cellular suspension of *Shewanella massilia* to the reaction mixture. Lanes M: size markers.

– Figure 17 shows electrophoresis on agarose gel (2%) of the products from PCR when amplification is effected starting from total DNA extracted from decomposing fish flesh (cod: lane 1 to 3; sole: lane 4 to 6; sea bream: lane 7 to 9, and surmullet: lane 10 to 12) using BN6+/BN4- primers (lanes 1, 4, 7 and 10, expected size: 364 bp), BN6+/BN2- primers (lanes 2, 5, 8 and 11, expected size: 711 bp), DDN1+/DDN5- primers (lanes 3, 6, 9 and 12, expected size: 820 bp). Lanes M: size standard (scale 1 Kb of BRL).

– Figure 18 shows electrophoresis on agarose gel (2%) of the products from PCR when amplification is effected using BC1+/BC2- molecular primers on two different preparations of total DNA from putrefying surmullet (lane 1 and 2). The arrow indicates the amplified DNA fragment of 177 base pairs.

– Figure 19 shows the alignment of the nucleotide sequences of the *tor*A genes of *Photobacterium phosphoreum* (torA/P.p.), of *Shewanella putrefaciens* (torA/S.p.), of *Shewanella massilia* (torA/S.m.), of *Shewanella c* (torA/S.c.), and of *Salmonella typhimurium* (torA/S.t.).

A) METHODS OF INVESTIGATION

Preparation of specific primers starting from the gene coding for the enzyme TMAO reductase: "DDN", "BN" and "BC" molecular probes.

The first step in the investigation described below relates to the preparation of molecular primers (or probes) that can be used for a PCR reaction, and to investigation of their specificity for different bacteria involved in the spoilage of fish flesh. The second step relates to application of the PCR technique to fish flesh.

I) Preparation and validation of molecular primers for use in PCR.

The choice of the nucleotide primers is a critical step for the success of a PCR test. These primers must be prepared starting from highly conserved regions of the gene

that codes for TMAO reductase in all of the bacteria responsible for the degradation of fish flesh. Several strategies have been employed for preparing these primers.

(1) Molecular primers prepared starting from regions of the *torA* gene conserved in bacteria of the genus *Shewanella* and in *E. coli*: "DDN" primers.

Production of primers with very little degeneration starting from conserved protein regions is very difficult owing to degeneration of the genetic code.

Molecular primers, called "DDN", were defined on the basis of the alignment of nucleic sequences of the *torA* gene of various bacteria of the genus *Shewanella* and of *E. coli* (Figure 9). In fact, several DNA regions of the *torA* gene in these bacteria have a high degree of sequence identity with very few mispairings.

The DDN molecular primers are the molecular primers DDN1+, DDN2-, DDN3-, DDN4-, DDN5- and DDN5+ as defined above.

For all of the PCR reactions, the pairing temperature is adjusted to 55°C in order to make amplification more specific and hence limit the appearance of contaminating DNA bands. This is made possible by the slight degeneration of the newly synthesized primers. PCR amplification employs 30 cycles of successive reactions. Each cycle comprises a step of denaturation at 94°C for 30 s, a hybridization step at 45°C for 30 s and an extension step at 72°C for 45 s.

a) Using DDN molecular primers for detecting the TMAO reductase system of bacteria of the genus *Shewanella*.

The first series of tests, using primer pairs DDN1+/DDN5-, DDN5+/DDN2-, DDN5+/DDN3-, DDN5+/DDN4-, was carried out on chromosomal DNA from the strain *Shewanella massilia* (*S. massilia*). The primer pairs DDN1+/DDN2-, DDN1+/DDN3- and DDN1+/DDN4- were deliberately discarded owing to the remoteness of their positions on the *torA* gene.

The results, summarized in Figure 10 (lanes 1 to 4), reveal for the majority of the pairs of molecular primers used, amplification of a unique DNA fragment specific to the expected size. Although an additional, nonspecific DNA fragment of about 300 base pairs is amplified for the primer pair DDN5+/DDN2- (lane 2), the DDN molecular primers prove to be specific for the TMAO reductase system of *S. massilia*.

The DDN primer pairs were then tested on other bacteria belonging to the *Shewanella* genus. The same series of tests was performed using, as substrate, the chromosomal DNA of the strain *Shewanella c* and *S. putrefaciens* MR-1. All of the

primer pairs make it possible to amplify a DNA fragment specific to the expected size for these two bacteria (Figure 10).

The various DDN primer pairs therefore permit detection of all of the *Shewanella* bacteria tested.

b) Using DDN molecular primers for detecting the gene coding for TMAO reductase in the marine bacterium *Photobacterium phosphoreum*, and for application of sequencing of the said gene.

Photobacterium phosphoreum (*P. phosphoreum*) is a bacterium which, like *S. putrefaciens*, is in a certain number of cases held responsible for the spoilage of the flesh of marine fish (12, 21). It belongs to the family *Vibrionaceae*, and its optimum growth temperature is 15°C. It is a bacterium that has received very little study, whose putative TMAO reductase system is absolutely unknown at present.

In order to verify the specificity of the molecular primers according to the invention on separate bacteria in the *Shewanella* group, which may contaminate fish flesh, a collection strain *P. phosphoreum* was obtained by the ATCC (American Type Culture Collection No. 11040). The first growth tests performed on this bacterium in marine medium (Difco) at 15°C showed that addition of TMAO to the culture medium significantly improves its growth rate. The optical density (OD_{600}) obtained after 24 hours of growth is 0.28 for the strain grown in the absence of TMAO, and 0.47 for the strain grown in the presence of TMAO. This result accords with the presence of a respiratory TMAO reductase in *P. phosphoreum*. Gene amplification was effected starting from the chromosomal DNA of this bacterium (prepared from 10 ml of culture in marine medium) using all of the pairs of DDN molecular primers.

The results, presented in Figure 11, show that the DDN primers permit amplification of a specific DNA fragment in all cases. The different sizes of DNA fragments accord with those obtained for amplification effected on the chromosome of bacteria of the *Shewanella* genus.

In order to confirm that the DNA fragment amplified in *P. phosphoreum* does correspond to a part of the gene coding for the TMAO reductase of *P. phosphoreum*, sequencing of the said fragment was undertaken. Thus, about 600 nucleotides of the fragment amplified starting from the pair of molecular primers DDN1+/DDN5- were sequenced. The PCR product directly purified by Geneclean (Bio 101) was sequenced by the chain termination technique (22) using the DDN1+ molecular primer. The protein sequence deduced from this nucleic sequence was aligned with those of various

TMAO reductases (Figure 7). This protein sequence displays approx. 60% identity with a region of the TMAO reductase of *S. massilia*. The homologies observed are high enough to conclude that the sequenced DNA fragment does correspond to a part of the gene that codes for the TMAO reductase of *P. phosphoreum*.

These results demonstrate the presence of a TMAO reductase system in the bacterium *P. phosphoreum*, and validate the method of detection according to the invention by the PCR test, since the latter makes it possible to detect the TMAO-reductase system in spoilage bacteria that are relatively distant phylogenetically.

c) Using DDN molecular primers for detecting genes coding for TMAO reductase of enterobacteria, and for implementing sequencing of the said genes.

In order to verify that the PCR test is valid for the detection of enterobacteria such as *E. coli* or certain pathogenic bacteria such as *Salmonella typhimurium*, the same PCR test as that described above was performed, with chromosomal DNA of *E. coli* and *S. typhimurium* as the substrate DNA.

In *S. typhimurium*, the promoting region of the TMAO reductase system has been demonstrated using a PCR approach (23).

The results obtained are shown in Figure 12. The four pairs of molecular primers DDN1+/DDN5-, DDN5+/DDN2-, DDN5+/DDN3- and DDN5+/DDN4- permit amplification of a specific DNA fragment in *E. coli* (lane 2, 6, 10 and 16). In the case of *S. typhimurium*, the pair of primers DDN1+/DDN5- also permits amplification of a DNA fragment, of 820 base pairs, compatible with a specific fragment of the TMAO reductase system (lane 3).

In order to confirm that the DNA fragment amplified in *S. typhimurium* corresponds well to a part of the gene coding for the TMAO reductase of *S. typhimurium*, sequencing of the said fragment was carried out. The protein sequence, deduced from a part of this DNA fragment (477 nucleotides), possesses important homologies with the protein sequence of various TMAO reductases (Figure 8). In addition it has more than 88% identity with the sequence of the TMAO reductase of *E. coli* and 45% with that of the enzyme in *S. massilia*. The DNA fragment amplified using DDN1+/DDN5- primers therefore probably corresponds to a part of the gene that codes for the TMAO reductase of *S. typhimurium*.

d) Conclusion

As described previously, the primer pair DDN1+/DDN5- permits detection of the *torA* gene of marine bacteria (*Shewanella* and *P. phosphoreum*), but also of enterobacteria (*E. coli* and *S. typhimurium*).

The same PCR tests were performed on bacteria isolated from brackish water (*Rhodobacter*), which might possibly be found in the flesh of fish. Samples of chromosomal DNA from the bacterium *R. sphaeroides* were employed as substrate DNA for performing these tests.

The bacterium *R. sphaeroides* possesses a DMSO/TMAO reductase system similar to that described in *E. coli* (9, 10). Although the terminal enzyme of this bacterium can reduce both TMAO and DMSO, it has numerous homologies of protein sequences with the TMAO reductase of *E. coli* or *S. massilia* (47.5% and 45% of identity, respectively).

However, the PCR tests conducted with the DDN molecular probes did not permit amplification of a DNA fragment corresponding to the gene of the TMAO reductase of this bacterium.

(2) Molecular primers prepared starting from regions of the *torA* gene conserved in all of the bacteria investigated: "BN" primers.

For further widening of the spectrum of detection of the molecular primers of the PCR test, the nucleic sequence of the gene of TMAO/DMSO reductase of bacteria of the genus *Rhodobacter* (*R. sphaeroides* and *R. capsulatus*) was integrated in the alignment performed starting from sequences of the *torA* gene of bacteria of the genus *Shewanella* and of *E. coli*.

A new series of primers, designated "BN", was prepared in this way (Figure 13).

For all the tests, PCR amplification is performed with a hybridization temperature of 55°C. At this temperature, just a small proportion of the primers present in the mixture will be able to hybridize specifically to the substrate DNA. For this reason, a larger quantity of molecular primers is added to the reaction mixture (about 100 pmol in final 50 µl).

The BN primers obtained are the primers BN1+, BN2-, BN3+, BN4-, BN5-, BN6+ as defined above.

Table 1 below shows the results obtained for application of the BN molecular primers on DNA from different bacteria.

Amplification is carried out starting from 5 ng of bacterial chromosomal DNA. The PCR reaction employs 30 successive reaction cycles.

+: A DNA fragment specific to the expected size is amplified

-: No amplification

Table 1

Bacteria	Primers BN1+/BN2-	Primers BN1+/BN4-	Primers BN3+/BN5-	Primers BN6+/BN4-	Primers BN6+/BN2-
S. massilia	+	+	+	+	+
Shewanella c	+	+	+	+	+
S. putrefaciens	+	+	+	+	+
P. phosphoreum	+	+	+	+	+
E. coli	+	-	+	+	+
S. typhimurium	-	-	+	+	+
R. sphaeroides	-	-	-	+	+

Two pairs of primers: BN6+/BN2- and BN6+/BN4-, permit amplification of a specific DNA fragment in all of the bacteria tested, and notably in *R. sphaeroides*.

The BN primers therefore prove to be perfectly appropriate for detecting bacteria that possess a TMAO reductase system, and that may contaminate fish flesh.

In addition, the use of a high hybridization temperature (55°C) during PCR, and a larger quantity of degenerated molecular primers, gave a significant improvement in the specificity of amplification.

(3) Molecular primers prepared starting from the gene coding for the TorC cytochrome of the TMAO reductase system: "BC" primers.

Investigation of the TMAO reductase system in various bacteria has demonstrated that the chain of electron transfer as far as the TorA terminal enzyme always involves a pentahaemic cytochrome of type *c*, the TorC (or DorC) cytochrome (6, 9, 10, 19).

In order to detect the TMAO reductase system in spoilage bacteria, nucleic primers directed against the gene coding for this cytochrome were prepared.

The multi-alignment of protein sequences, shown in Figure 14, reveals numerous protein regions conserved among the cytochromes of the TMAO reductase system of various bacteria: *S. massilia*, *E. coli*, *R. sphaeroides*. Some of these conserved regions

correspond to the sites of fixation of haems of type *c* (CxxCH motif) and so are found in a great many tetrahaemic cytochromes of class NapC/NirT involved in other respiratory systems (24). Nevertheless, the TorC cytochrome possesses the special feature that it contains an additional carboxy-terminal domain which fixes a fifth haem (6, 19).

The BC molecular primers are the primers BC1+, BC2+, BC2- and BC3- as defined above. To prepare the said primers, conserved protein regions were sought among all of the cytochromes of class NapC/NirT (probes BC2+ and BC2-), but also uniquely among the TorC cytochromes (probes BC1+ and BC3-). The position and orientation of the four degenerated molecular primers BC are indicated in Figure 14.

The BC molecular primers are capable of hybridizing to the gene coding for the TorC cytochrome of the TMAO reductase system.

The PCR reaction is carried out in the same conditions as those used with the BN molecular primers. It employs 30 successive reaction cycles. Each cycle consists of a step of denaturation at 94°C for 30 s, a hybridization step at 55°C for 30 s and an extension step for 45 s.

About 100 pmol of each molecular primer is added to the reaction mixture (final volume 50 µl). Among the three pairs of molecular primers tested (BC1+/BC2-, BC1+/BC3- and BC2+/BC3-), the pair BC1+/BC2- made it possible to amplify a DNA fragment to the expected size (177 base pairs) for all of the bacteria previously tested (Figure 15; lanes 1 to 5). This result enables us to envisage using the pair of molecular primers BC1+/BC2- for the PCR test.

In parallel, the same PCR test was performed with the primer pair BC1+/BC2- starting from the chromosome of the bacterium *Erwinia chrysanthemi*, a phytopathogenic enterobacterium that would lack a TMAO reductase system (4). In this case, no DNA fragment of a size compatible with that expected for amplification starting from the *torC* gene is observed (Figure 15, lane 6).

The primer pair BC1+/BC2- therefore seems to specifically recognize the TorC cytochrome of the TMAO reductase system.

(4) Application of the PCR technique to whole cells

For performing a PCR test that is intended to be used directly starting from fish samples, it is important to eliminate the step of preparation of the chromosomal DNA of the bacteria. For this reason, PCR tests using DDN, BN and BC primer pairs were reproduced starting from whole bacteria of *S. massilia*. Bacterial colonies isolated in a

dish are resuspended in 200 µl of sterile distilled water (OD_{600} between 0.1 and 0.5). The PCR reaction is then carried out in the conditions previously described with 2.5 µl of the cellular suspension as substrate (in 50 µl of final volume). For all of the PCR reactions performed (with DDN, BN and BC primers), the result obtained is equivalent to that observed with purified chromosomal DNA. The bacteria are therefore well lysed during the first step of the PCR which corresponds to incubation of the samples at 94°C for 1.5 min. On the basis of this positive result, we can envisage using the PCR test directly starting from fish flesh.

II) Application of the PCR-TMAO test to fish flesh

Having verified the specificity of the molecular primers according to the invention on the bacteria responsible for the spoilage of seafoods, the next step therefore consists of directly applying the PCR test to the flesh of fish.

The four primer pairs DDN1+/DDN5-, BN6+/BN4-, BN6+/BN2- and BC1+/BC2- were chosen for performing these tests because they permit amplification of a specific DNA fragment for the vast majority of the bacteria previously tested.

(1) Influence of fish chromosomal DNA during PCR amplification of the gene of TMAO reductase of bacteria.

For use of the PCR test on samples of fish flesh, it is first necessary to verify that the fish chromosomal DNA does not compete with the bacterial DNA during hybridization of the molecular primers.

In order to test the effect of fish chromosomal DNA on the specificity of the molecular primers according to the invention, variable concentrations of purified chromosomal DNA of herring sperm were added to the PCR reaction mixture with or without 2.5 µl of cellular suspension of the bacterium *S. massilia*.

Figure 16, obtained with the primer pair DDN1+/DDN5-, shows that no DNA fragment is amplified in the absence of bacterium added to the reaction mixture (lane 1 to 3), regardless of the concentration of fish chromosomal DNA. If the bacterium *S. massilia* is added to the reaction mixture (lanes 4 to 6) a specific DNA fragment is amplified. The same results were obtained for the primer pairs BN6+/BN2-, BN6+/BN4- or BC1+/BC2-, and so it can be concluded that the presence of foreign

DNA in the PCR reaction mixture does not interfere with the specificity of the molecular primers according to the invention.

On the basis of these important results it is therefore possible to exclude the occurrence of nonspecific amplification that would be due to the fish chromosomal DNA.

(2) Extraction of total DNA from fish flesh and validation of the PCR technique.

Traditionally, tests for detecting microorganisms during microbiological inspection of foodstuffs require a first step of pre-enrichment of the bacteria on a selective medium.

With a view to a quick test of freshness, the PCR technique was applied directly to fish samples. Various conditions of extraction of the total DNA from the flesh of putrefying fish (bacterial DNA + fish DNA) were therefore tested.

Firstly, a quick technique of DNA extraction from cells was tested by treating fish samples with NaOH/SDS (25). This technique permits cell lysis and therefore extraction of total chromosomal DNA. The sample was prepared from about 25 mg of flesh taken from a fish (surmullet) stored at room temperature for 12 hours. After incubation at 95°C for 10 min, 5 µl of the mixture of homogenized fish flesh was used directly in the PCR reaction mixture.

However, using this quick technique for extraction of total DNA, the full range of PCR reactions effected with the various molecular primers did not permit amplification of a specific DNA fragment. The presence of several PCR inhibiting substances contained in the fish flesh might explain this result. In fact, compounds that are present in large quantity in certain food products (fats, proteins, etc.) may affect the efficacy of PCR (26). In order to verify this hypothesis, the same PCR tests were conducted with addition of 5 ng of chromosomal DNA from *S. massilia* to the samples of fish flesh. As previously, however, no DNA fragment was amplified by PCR, regardless of which molecular primers were used.

It can be concluded from these results that the PCR reaction is inhibited by certain compounds contained in fish flesh.

The technique for purifying the total chromosomal DNA from fish flesh, employed according to the present invention, must therefore make it possible to eliminate all of the PCR inhibiting compounds.

A quick DNA extraction kit (about 1 hour), based on fixation of nucleic acids on silica beads, was therefore tested (kit marketed as "High Pure PCR Template Preparation Kit" by Boehringer Mannheim/Roche). After fixing the DNA, this technique actually makes it possible to eliminate the salts, proteins and other cellular impurities in a single step of washing.

Extraction of total DNA (bacterial DNA + fish DNA) was performed on four different species of fish undergoing decomposition (stored for 12 h at room temperature): 2 species from the Mediterranean Sea (surmullet and sea bream), and 2 species from the Atlantic (sole and cod). Starting with about 50 mg of flesh from these fish (taken from under the skin), 1 to 4 μg of total chromosomal DNA was purified in this way. About 25 ng of this DNA was then added to the PCR reaction mixture (final volume 50 μl). For all of the PCR reactions, the hybridization temperature is adjusted to 55°C, and 100 pmol of each molecular primer is added to the reaction mixture.

Figure 17 summarizes the results obtained using the primer pairs DDN1+/DDN5-, BN6+/BN2- and BN6+/BN4-. For all of the fish species tested, a unique DNA fragment is amplified, with each of the primer pairs used.

The size of the amplified DNA fragment corresponds to the size expected for amplification starting from the *torA* gene. This result shows that the various DNA fragments were indeed amplified starting from the *torA* gene of the bacteria present on the fish flesh.

Furthermore, tests that were carried out on another two species of fish undergoing putrefaction (7 days under melting ice), mackerel (fat fish) and whiting, detected the torA gene of the spoilage bacteria.

Thus, use of the pairs of molecular primers DDN1+/DDN5-, BN6+/BN2- and BN6+/BN4- can be envisaged for application of the PCR test to fish flesh.

Furthermore, the use of a silica bead filter is a suitable method for purifying the total DNA contained in fish flesh. This technique is very fast (about one hour), and very easy to implement in comparison with isoamyl alcohol/chloroform extraction, which requires several steps for eliminating the proteins contained in fish flesh (28).

Amplification effected in the same conditions with the primer pair BC1+/BC2- also gave encouraging results for total DNA from flesh of surmullet undergoing putrefaction (Figure 18). In fact, despite considerable background noise, agarose gel electrophoresis of the PCR products reveals the presence of the 177 bp fragment.

Application of the technique for monitoring spoilage of cod demonstrated that the technique is in direct correlation with the freshness quality of fish flesh during storage over time.

III) Conclusion

The pairs of molecular primers according to the invention therefore make it possible to detect the *torA* gene in marine bacteria, as well as in bacteria that are relatively remote phylogenetically, namely enterobacteria and bacteria from brackish water.

Application of the PCR test to fish flesh gives encouraging results since the molecular primers according to the invention permit detection of the *torA* gene of bacteria present on putrefying fish. PCR amplification cannot be applied directly to samples of fish flesh, so extraction of the total DNA from the said flesh is required first.

Furthermore, the technique is directly related to the freshness of fish flesh and is more sensitive than the classical techniques such as TVBN.

B) MATERIALS AND METHODS

1) Isolation of the bacterial strains; growth media and conditions

The fish used for isolating the bacterial strains *Shewanella c* and *Shewanella massilia* corresponds to a surmullet (*Mullus surmuletus*) caught in the Mediterranean Sea off Marseilles, and kept at room temperature for 48 hours in a sterile tube containing sea water. The sea water had been sterilized beforehand through a millipore filter (0.45 µM).

The *Shewanella* strains are cultivated in anaerobic or aerobic conditions at 30°C in LB medium ("Luria Broth": yeast extract 10 g/l, bacto-peptone 5 g/l and NaCl 5 g/l). The bacteria *E. coli* and *Salmonella typhimurium* are cultivated at 37°C on LB medium. The strain *Photobacterium phosphoreum* was obtained by the ATCC (No. 11040) and does not grow on conventional media such as the LB medium. It is cultivated at 15°C on marine medium (marine broth; Difco).

2) PCR techniques

a) Standard PCRs

Standard PCR amplifications are conducted in the conditions described in reference (6). The reaction mixture (50 µl) contains 10 ng of chromosomal DNA, 0.2 µg

of each of the oligonucleotide probes, 100 µM of each of the four triphosphate deoxyribonucleotides (dXTPs), 10 mM of Tris/HCl (pH 8.3), 1.5 mM of $MgCl_2$, 50 mM of KCl, 0.01% of gelatin and one unit of DNA polymerase (Taq polymerase, Boehringer Mannheim/Roche). The reaction mixture is finally covered with 50 µl of mineral oil to prevent evaporation during the PCR process.

Amplification, carried out in a thermocycler (MJ Research), employs 30 cycles of reactions, one cycle comprising a step of denaturation at 94°C for 30 seconds, a step of hybridization at 55°C for 30 seconds and a step of extension at 72°C for about 45 seconds (1 kb/min). The DNA is denatured beforehand for 1.5 min at 94°C before commencing the first cycle. 10 µl of each of the amplification products is then analysed by agarose gel electrophoresis.

b) PCR conditions with the degenerated molecular primers according to the invention

The molecular primers according to the invention, especially the primers designated "DDN", "BN" or "BC", are as defined above.

The reaction mixture (50 µl) contains either 10 ng of chromosomal DNA from the bacterium, or 5 µl of cellular suspension (OD_{600} = 0.5-1) or 25 ng of total DNA extracted from fish, 100 µM of each of the four triphosphate deoxyribonucleotides (dXTPs), 0.8 µg of each of the mixtures of primers according to the invention, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% of gelatin and one unit of Taq polymerase.

The PCR reaction employs 30 successive reaction cycles comprising a step of denaturation at 94°C for 30 seconds followed by a step of hybridization at 55°C or 45°C for 30 seconds, then a step of extension at 72°C for 45 seconds. The DNA is denatured beforehand for 1.5 min at 94°C before commencing the first cycle.

3) Preparation of chromosomal DNA from bacteria

The chromosomal DNA from bacteria was prepared starting from 10 ml of culture (OD_{600} > 1). After centrifugation of the cells at 10 000 rpm for 10 min, the residue is resuspended in 1 ml of EDTA 10 mM (pH 8). Cell lysis is achieved by adding SDS (0.5% final). This solution is mixed with an equal volume of isoamyl alcohol/chloroform (1:24). After centrifugation for 20 min at 7000 rpm, the top phase is

recovered. This operation is repeated several times in order to eliminate most of the residual proteins. Finally the DNA is precipitated by adding NaCl (0.3 M final) and ethanol (2.2 Vol).

4) Preparation of total chromosomal DNA from putrefying fish flesh for PCR

a) Quick preparation using a kit based on DNA fixation to silica beads (High pure PCR template Preparation Kit, Boehringer Mannheim/Roche)

25 mg of fish flesh is incubated for 45 min at 55°C in 200 µl of lysis buffer (urea 4 M, Tris 200 mM, NaCl 20 mM and EDTA 200 mM, pH 7.4) in the presence of 40 µl of proteinase K (0.8 mg). To facilitate cell lysis, the sample is first broken up using a scalpel. 200 µl of fixation buffer (guanidine-HCl 6 M, urea 10 mM, Tris-HCl 10 mM and Triton 20% v/v, pH 4.4) is added to the sample which is then incubated at 72°C for 10 min. This solution is mixed with 100 µl of isopropanol then centrifuged at 8000 rpm for 1 min through a filter based on silica beads (tube High Pure Filter + collecting tube). The residual impurities are eliminated in two washing steps (washing buffer: NaCl 20 mM, Tris-HCl 2 mM, ethanol 80% v/v, pH 7.5). The DNA is then eluted in an elution buffer (Tris 10 mM, pH 8.5).

b) Extraction of DNA from cells by treatment with NaOH/SDS

25 mg of fish flesh is taken from a putrefying fish (after incubation for 16 hours in a sterile tube kept at room temperature), and placed in 50 µl of a solution of NaOH 0.05 M, SDS 0.25%. After grinding the cells, the sample is incubated at 95°C for 15 min. 450 µl of sterile water is added to the mixture and the cell debris is then eliminated in a centrifugation step of 2 min at 8000 g. 5 µl of the supernatant obtained is used directly for the PCR test.

c) Isoamyl alcohol/chloroform extraction

Preparation of total chromosomal DNA by isoamyl alcohol/chloroform extraction is carried out starting from 25 mg of contaminated fish flesh as described previously for bacterial DNA. However, the number of extraction steps is increased so as to be able to eliminate the large quantities of impurities contained in the samples of fish flesh.

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